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14. ABSTRACT

Epigenetic gene silencing is a common element of breast cancers. The specific genes targeted and the mechanisms of silencing, and how these relate to disease progression and outcome, are not well understood. This research project has 1) identified a new breast tumor suppressor gene (RIN1) that is silenced in the majority of breast tumor cell lines and most tumor tissue samples tested; 2) begun to describe a breast tumor suppressor locus with three genes (B3GNT6, BRMS1, RIN1) that appear to be coordinately silenced in breast cancer; 3) uncovered multiple mechanisms of gene silencing operating at this locus and 4) demonstrated the development of specific tumorigenic phenotypes resulting from loss of RIN1 expression.

15. SUBJECT TERMS
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Introduction

Breast cancer initiation and progression reflect a mix of genetic events that include gain of function and loss of function mutations in genes regulating various cell division and cell death pathways. In addition to these alterations, several epigenetic changes are believed to contribute to the phenotypes of breast cancer cells. Recent models for breast cancer have evolved to highlight the diversity of genetic alterations (e.g. ER expression, ERBB2 or EGFR overexpression, TP53 mutation or deletion) that define distinguishable tumor subtypes with characteristic properties, prognoses and responsiveness to therapeutic interventions. However, less is known about the epigenetic events leading to gene silencing of breast tumor suppressor genes. The full panel of breast tumor suppressor genes has not yet been identified, and for many of the known tumor suppressors their role in cell transformation has not been elucidated. In addition, our understanding of gene silencing mechanisms is still inadequate. These novel tumor suppressor genes, and the mechanisms that regulate them, are prime targets for new therapeutic approaches.

Body

This project is focused on the determination whether RIN1, a gene encoding a RAS effector protein, is a breast tumor suppressor gene. We have taken multiple approaches to answer this question. Employing Real Time PCR (RT-PCR) we have made quantitative measures of RIN1 expression in a panel of breat tumor cell lines. Using laser capture microscopy, we have also measured RIN1 expression in tumor and surrounding normal tissue from breast cancer patient samples. This ensures that the silencing of RIN1 that we have observed is not simply the result of events occuring in culture systems.

Intriguingly, the RIN1 gene is tightly linked to BRMS1 (Breast Tumor Metastasis Supressor 1), which encodes a subunit of a chromatin remodeling complex (1, 2), and the B3GNT6 gene, which encodes a protein glycosylation enzyme (3) of a type that has been implicated in transformation and cancer. Each of the three genes is separated by less than 1 kb, and each is transcribed in the same direction. As described below, we observed that all three genes are coordinately repressed in many breast tumor cell lines, although RIN1 is the most strongly silenced. These data have led us to speculate that the B3GNT6-BRMS1-RIN1 cluster may be co-regulated during development and may represent a breast tumor suppressor locus. This arrangement has not been previously described and could provide novel insights into the connection between developmental programs and tumorigenesis in breast tissue.

Our findings also suggest that RIN1 silencing results form different mechanisms in different tumors. This was an unexpected result, as tumor suppressor genes are typically thought to be shut off by a single type of event (mutation/deletion, gene methylation or transcription repressor overexpression).

To evaluate the phenotypic changes cause by loss of RIN1 expression, we have carried out experiments using mammary epithelial cells (MECs) from *Rin1*$^{-/-}$ mutant mice and control wild type mice (4). We observed three phenotypic changes that directly connect loss of RIN1 with altered cytoskeleton dynamics and cellular transformation. Compared to wild type MECs, the *Rin1*$^{-/-}$ MECs showed enhanced attachment to fibronectin, elevated rates of cell motility, and retarded formation of cell-cell contacts. These results are described in detail in *Current Biology* 15, 815-823 (see appendix).

Key Research Accomplishments

We can report specific accomplishements in multiple areas of this project. We have demonstrated that RIN1 silencing is widespread among breast tumor cell lines (all nine lines tested) and in tumors (all four patient samples examined). This widespread repression of a RAS effector gene is likely to play an important role in understanding breast tumor etiology. Another major finding is that B3GNT6 and BRMS1, genes found immediately upstream of RIN1, are coordinately silenced in many tumor cell lines. This observation opens a new avenue for evaluating the contribution of multiple linked genes to mammary cell biology and tumorigenesis. This challenges the more common view that the silencing of neighbor genes is simply a bystander effect secondary to repression of a single tumor suppressor gene.

Our findings during this funding period also demonstrate that multiple mechanisms of gene silencing can operate at the same locus in independent tumors. Specifically, we have shown that silencing of the RIN1 locus can be induced by methylation (reversible by treatment with a demethylating agent) or by overexpression of SNAI1 (reversible by si-RNA mediated knockdown). We are currently performing experiments to evaluate whether these are direct or indirect effects.

Using a mouse model system, we have demonstrated how the loss of RIN1 expression significantly alters mammary epithelial cells, confering a mesenchymal-like phenotype characterized by increased adhesion to fibronectin, enhanced motility and reduced ability to form contiguous epithelial sheets. These findings suggest a direct relationship between RIN1 function and epithelial-mesenchymal transition (EMT). Because RIN1 is a downstream effector of RAS, our results also suggest a previously unappreciated connection between RAS signaling and EMT. This connection may be through the ABL tyrosine kinases, which are activated by RIN1 binding, or by signaling contributions from RAB5, which is activated by a guanine nucleotide exchange factor domain within RIN1.

Reportable Outcomes

1. The RIN1 gene is silenced by 90% or greater in nine breast tumor cell lines examined.
2. RIN1 is silenced in four tumor tissues, compared to surrounding normal mammary epithelial cells.
3. The tightly clustered three-gene locus B3GNT6-BRMS1-RIN1 is coordinately silenced in multiple breast tumor cell lines and primary tumors.
4. In some cases, RIN1 appears to be silenced either directly or indirectly through methylation, and gene expression can be restored by treatment with the demethylation drug Zebularine.
5. In some other cases, RIN1 appears to be silenced either directly or indirectly through elevated expression of the transcriptional repressor SNAIL (SNAI1), and gene expression can be restored by treatment with siRNA targeted to SNAIL.
6. The loss of RIN1 expression in mouse mammary epithelial cells (MECs), derived from a gene disruption mouse model previously generated in this lab, demostrates that RIN1 normally inhibits EMT transition (i.e. RIN1 loss promotes cell attachement and migration while retarding the formation of epithelial sheets).

Conclusions

Several conclusions can be derived from this research. First, RIN1 is strongly and consistantly silenced in human breast tumor cell lines and primary tumors, implying a tumor suppressor role for this RAS effector. Second, RIN1 is part of a tightly linked and coordinately regulated three-gene locus (B3GNT6-BRMS1-RIN1) on chromosome 11. Third, gene silencing can occur at the same locus through different mechanisms in individual tumors.

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Appendices

Hu, H. Bliss, J.M., Wang, Y. and Colicelli, J. (2005) RIN1 is an ABL Tyrosine Kinase Activator and a Regulator of Epithelial Cell Adhesion and Migration. *Curr. Biol.* 15: 815-823.
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RIN1 Is an ABL Tyrosine Kinase Activator and a Regulator of Epithelial-Cell Adhesion and Migration

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Summary

Background: ABL tyrosine kinases control actin remodeling in development and in response to environmental stimuli. These changes affect cell adhesion, cell migration, and cell-cell contact. Little is known, however, about upstream mechanisms regulating ABL protein activation.

Results: We report that the RAS effector RIN1 is an activator of ABL tyrosine kinases. RIN1 expression in fibroblasts promotes the formation of membrane spikes; similar effects have been reported for ABL overexpression. RIN1 binds to the ABL SH3 and SH2 domains, and these interactions stimulate ABL2 catalytic activity. This leads to increased phosphorylation of CRK and CRKL, inhibiting these cytoskeletal regulators by promoting intramolecular over intermolecular associations. Activated RAS participates in a stable RAS-RIN1-ABL2 complex and stimulates the tyrosine kinase-activation function of RIN1. Deletion of the RAS binding domain (RBD) strongly stimulated the ABL2 activation function of RIN1, suggesting that RAS activation results from the relief of RIN1 autoinhibition. The ABL binding domain of RIN1 (RIN1-ABD) increased the activity of ABL2 immune complexes and purified RIN1-ABD-stimulated ABL2 kinase activity toward CRK. Mammary epithelial cells (MECs) from *Rin1*$^{-/-}$ mice showed accelerated cell adhesion and increased motility in comparison to wild-type cells. Knockdown of RIN1 in epithelial-cell lines blocked the induction of CRKL phosphorylation, confirming that RIN1 normally functions as an inhibitor of cell motility.

Conclusions: RIN1 is a directly binding ABL tyrosine kinase activator in cells as well as in a defined-component assay. In response to environmental changes, this novel signal pathway mediates actin remodeling associated with adhesion and migration of epithelial cells.

Introduction

The nonreceptor tyrosine kinases ABL1 (also known as c-Abl) and ABL2 (also known as Arg) are similar in structure and function [1]. Both proteins contain SH3, SH2, and tyrosine kinase (TK) domains; in each case, the domains exhibit >90% sequence identity. Both ABL proteins also have leukemogenic potential that is unleashed by chromosome translocations [2–4]. The resulting oncogenic fusion proteins always include the SH3, SH2, and TK domains.

ABL proteins regulate cytoskeleton remodeling (reviewed in [5, 6]) during adhesion, motility, and axon guidance. Consistent with this function, ABL proteins contain actin binding sites [7–9], localize to dynamic actin structures, and phosphorylate CRK and CRKL [10, 11], DOK1 [12], and other proteins controlling cytoskeleton dynamics [13]. Tyrosine phosphorylation of CRK/CRKL promotes intramolecular refolding that excludes interactions with CAS and blocks cell migration (reviewed in [14]).

Inactive ABL kinases are stabilized by intramolecular interactions including the following: (1) association between the SH3 domain and the SH2-TK linker region [15], (2) interactions of a short amino-terminal "cap" [16], and (3) contributions from an amino-terminal myristoyl group [17] and phospholipids [18]. ABL kinase activation is facilitated by autophosphorylation as well as by SRC-family kinase-mediated phosphorylation [19–21]. Intermolecular ABL binding partners that inhibit function include Abi1 [22], Abi2 [23], AAP1 [24], PAG [25], and RB1 [26]. Although overexpression of some genes can promote phosphorylation of ABL substrates in vivo [27, 28], there are no reports of direct ABL kinase activators.

RIN1 is a RAS-effector protein [29, 30] that binds to ABL1 [29] and BCR-ABL1 [31]. The RIN1-ABL1 interaction appears to involve the SH3 and SH2 domains of ABL1 [31, 32], but the mechanism of binding and the consequences of this association were previously not known. The expression of RIN1 protein is restricted [33] but includes some epithelial cells.

We report that RIN1 is a binding partner for ABL2 as well as for ABL1, that RIN1 phosphorylation by ABL is required for stable binding, that RIN1 functions to stimulate ABL2 activity in a RAS-responsive manner, and that the ABL binding domain of RIN1 can directly enhance ABL2-mediated phosphorylation of CRK. We also show that RIN1 promotes the cytoskeletal-remodeling properties of ABL proteins and serves in part to regulate epithelial-cell functions including adhesion and migration.

Results

RIN1 Functions through ABL Kinases to Induce Actin Remodeling

We introduced RIN1 into fibroblast cells, which normally have undetectable levels of this protein. When plated on fibronectin, RIN1-expressing cells showed a striking change in morphology (Figure 1A). We noted the appearance of microspike structures and a decrease in actin stress fibers, similar to changes seen after overexpression of ABL1 [12, 34]. RIN1-expressing cells also had a rounded-up morphology (Figure 1B; cell height: 8.3 ± 0.6 μm [vector], 14.8 ± 1.2 μm [RIN1]). Microspike formation was also observed when we expressed the ABL binding domain alone (RIN1-ABD, Fig-

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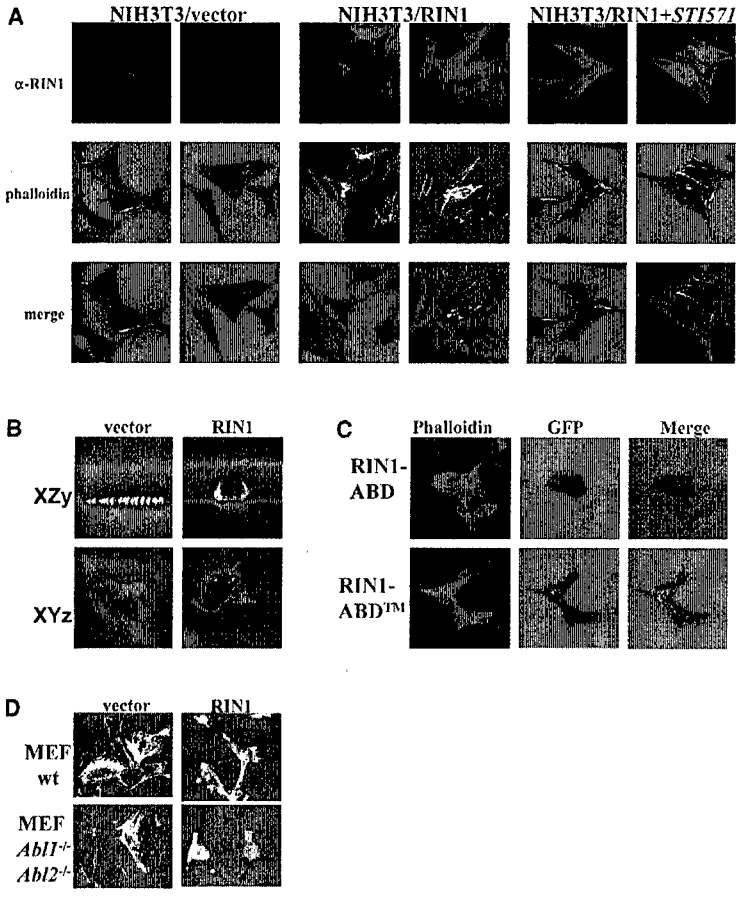

Figure 1. RIN1 Expression Promotes ABL-Dependent Actin Remodeling in Fibroblasts

(A) NIH3T3 cells, infected with a RIN1 or vector retrovirus and stained with phalloidin (green) and anti-RIN1 (red). RIN1 effects were blocked by STI571.

(B) At top, XZy confocal analysis, phalloidin (green) stain; at bottom, XYz confocal analysis and phalloidin (green) and anti-RIN1 (red) staining of the same cells as on top.

(C) NIH3T3 cells expressing the ABL binding domain alone (RIN1-ABD) or the triple Y/F mutation (RIN1-ABD™) from a green-fluorescent-protein (GFP) vector. The phalloidin stain was red in this experiment.

(D) MEFs, infected with a RIN1 or vector retrovirus and stained with phalloidin (green). RIN1 induced membrane spikes in wild-type but not in *Abl1*$^{-/-}$*Abl2*$^{-/-}$ MEFs.

ure 1C). These results are consistent with RIN1 activation of an ABL-mediated pathway. Treatment with STI571, a potent inhibitor of both ABL1 and ABL2, effectively blocked cell remodeling (Figure 1A) but had no effect on the morphology of NIH3T3 cells not expressing RIN1 (data not shown). To confirm that RIN1-induced remodeling was ABL dependent, we compared the effects of RIN1 expression in mouse embryo fibroblast (MEF) cells from wild-type and *Abl1*$^{-/-}$*Abl2*$^{-/-}$ animals [35]. The absence of ABL1 and ABL2 blocked the ability of RIN1 to induce cell shape changes (Figure 1D), although these cells remain responsive to ABL (Figure S1 in the Supplemental Data available with this article online).

RIN1 Is a Binding Partner of ABL Tyrosine Kinases

When ABL2 was immunoprecipitated from cell extracts, we observed copurification of RIN1 (Figure 2A, left), indicating a stable protein complex. This was confirmed by reciprocal analysis (RIN1 immunoprecipitation followed by ABL2 immunoblot; Figure 2A, right). We also detected an interaction between ectopically expressed RIN1 and ABL1 (Figure 2B, left) as well as between endogenous epithelial-cell RIN1 and ABL1 (Figure 2B, right). The RIN1-ABL1 coimmunoprecipitation appeared weaker than that of RIN1-ABL2, perhaps reflecting ABL1 distribution in both the cytoplasm and the nucleus.

Because RIN1 is phosphorylated by ABL1 in vitro [29] and by BCR-ABL1 in vivo [31], we tested whether RIN1 is subject to tyrosine phosphorylation by endogenous ABL proteins and whether this was required for RIN1-ABL binding. RIN1 ectopically expressed in wild-type, *Abl1*$^{-/-}$, or *Abl2*$^{-/-}$ MEF cells was tyrosine phosphorylated (Figure 3A), but RIN1 expressed in *Abl1*$^{-/-}$*Abl2*$^{-/-}$ cells had no detectable tyrosine phosphorylation. These findings suggested that RIN1 is phosphorylated by both ABL1 and ABL2. Because RIN1 tyrosine 36 conforms to both the general tyrosine-phosphorylation consensus (R/K-X_{2-3}-D/E-X_{2-3}-Y) and the ABL substrate motif (YxxP), we developed a phosphospecific antibody to monitor RIN1 phosphorylation by ABL1 and ABL2. Anti-RIN1pY^{36} was highly specific for tyrosine-phosphorylated RIN1 in cells coexpressing ABL2 (Figure 3B). In addition, the antibody recognized endogenous RIN1pY^{36} in KCL22 cells, a leukemia-derived cell line expressing BCR-ABL1 (Figure 3B), and in forebrain tissue, which has high levels of ABL2 and RIN1 [33, 35]. The identified RIN1pY^{36} band was absent from *Rin1*$^{-/-}$ tissue and showed reduced intensity in extracts from *Abl2*$^{-/-}$ forebrains (Figure 3C).

We next tested whether phosphorylation by ABL proteins is required for RIN1 binding. When cotransfected with RIN1, a catalytically inactive form of ABL2 (ABL2^{K319R}) gave only low levels of RIN1pY^{36} and weak

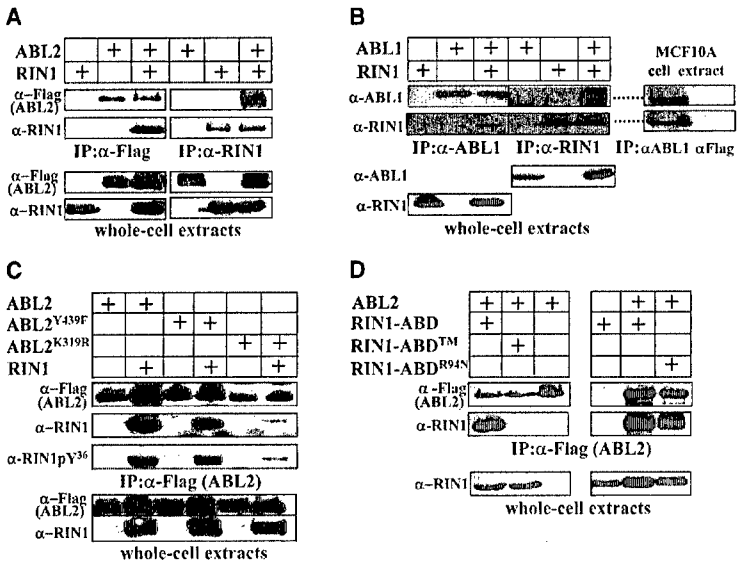

Figure 2. RIN1 Is Associated with ABL Proteins

(A) Anti-Flag (ABL2)-immunoprecipitated material was immunobloted with anti-RIN1 (left), and anti-RIN1-immunoprecipitated material was immunobloted with anti-Flag (right). These immunoblots used extracts from transfected 293T cells.

(B) RIN1 binding to ABL1 was assessed with anti-RIN1 or anti-ABL1 immunoprecipitation from extracts of transfected 293 cells. The right side shows immunoprecipitation of endogenous RIN1 with endogenous ABL1 from MCF10A cells when anti-ABL1 but not anti-Flag (control) was used.

(C) RIN1 tyrosine phosphorylation is required for ABL2 binding. RIN1 was efficiently coimmunoprecipitated with wild-type ABL2-Flag or a phosphorylation-site mutant ($ABL2^{Y439F}$) but not with a kinase inactive mutant ($ABL2^{K319R}$).

(D) RIN1 tyrosine phosphorylation is required for ABL2 binding. RIN1-ABD and an SH2 mutant ($RIN1\text{-}ABD^{R94N}$), but not a triple-tyrosine mutant ($RIN1\text{-}ABD^{TM}$), coimmunoprecipitated with ABL2-Flag. Whole-cell-extract immunoblots were used to confirm expression of transfected constructs.

ABL2-RIN1 binding (Figure 2C). The detectable $RIN1pY^{36}$ likely reflects the action of endogenous ABL1 and ABL2. Mutation of an activation-loop phosphorylation site (Y439) required for optimum kinase activity [21] had no effect on RIN1 phosphorylation or binding (Figure 2C).

We confirmed the requirement for RIN1 tyrosine phosphorylation in ABL2 binding through the use of a mutant form of the ABL binding domain (ABD). $RIN1\text{-}ABD^{TM}$ has three tyrosines mutated to phenylalanines (in the absence of Y^{36}, two other tyrosines within the ABD [Y^{121} and Y^{148}] are phosphorylated by BCR-ABL1 [31]). $RIN1\text{-}ABD^{TM}$ was impaired for binding to ABL2 (Figure 2D) and failed to induce membrane spikes in NIH3T3 cells (Figure 1C). A mutation (R94N) in the RIN1 SH2 domain, which mediates receptor tyrosine kinase interactions [36], had no effect on ABL binding.

These results are consistent with a protein-interaction model that is initiated by a weak association between the RIN1 proline-rich motif and the ABL SH3 domain and is followed by tyrosine phosphorylation of RIN1. Subsequent connection of this phosphotyrosine to the ABL SH2 domain produces a stable two-site association.

Stimulation of ABL2 Tyrosine Kinase Activity by RIN1

To determine whether RIN1 could influence the catalytic activity of ABL proteins, we examined cellular tyrosine-phosphorylation levels. Autophosphorylated ABL2 protein was easily detectable, and this was noticeably elevated when RIN1 was coexpressed in cells (Figure 4A). Phosphorylated RIN1 was also clearly evident (293T cells have extremely low levels of endogenous RIN1 [data not shown]). Several additional, unidentified bands also appeared in extracts coexpressing ABL2 and RIN1, indicating a broader induction of tyrosine phosphorylation. The new phosphotyrosine bands most likely reflect ABL2 stimulation because these bands were absent in extracts from cells transfected with $ABL2^{K319R}$, the kinase dead mutant (Figure 4A). We also found that RIN1 expression increased cellular tyrosine phosphorylation by $ABL2^{Y439F}$, a regulatory phosphorylation-site mutant of ABL2 (Figure 4A), providing evi-

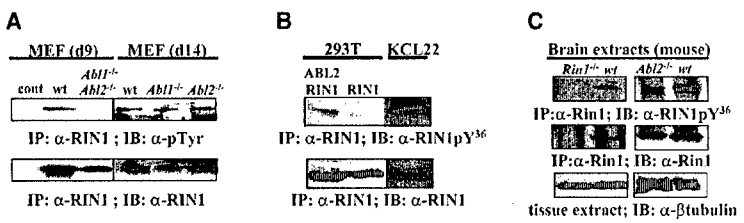

Figure 3. RIN1 Is Phosphorylated by ABL Kinases

(A) RIN1 was introduced into MEF cells and analyzed by anti-RIN1 immunoprecipitation followed by anti (α)-pTyr immunoblot (upper panel) or anti (α)-RIN1 immunoblot (lower panel). For $Abl1^{-/-}Abl2^{-/-}$ cells, matched day-9 MEFs were used because of early lethality [34]. Control cells were not infected with RIN1.

(B) Detection of ectopic phospho-RIN1 in transfected 293T cells (left) and endogenous phospho-RIN1 in BCR-ABL1-positive KCL22 cells (right) with anti-$RIN1pY^{36}$.

(C) Detection of endogenous $RIN1pY^{36}$ in forebrain tissue extracts prepared from adult mice of the indicated genotype. Rin1 was immunoprecipitated with anti-Rin1 and analyzed by immunoblot with anti-$RIN1pY^{36}$ or anti-Rin1. Brain extracts were normalized with an anti-β tubulin probe prior to immunoprecipitations.

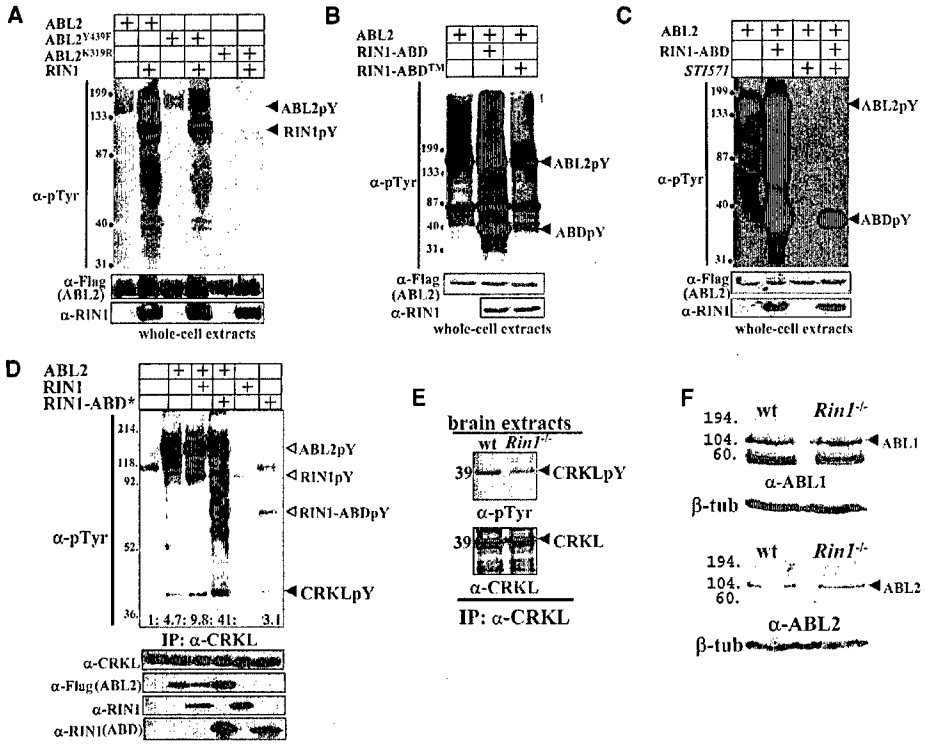

Figure 4. RIN1 Promotes ABL2 Kinase Activity

(A and B) Transfected 293T cell lysates were analyzed by immunoblot with anti-phosphotyrosine. ABL2 and RIN1 expression were confirmed by separate immunoblots (bottom panels). Note that in panel (B), gradient PAGE led to altered protein mobility.

(C) Analysis of cellular phosphotyrosine after treatment of cells with STI571 (10 μM).

(D) Endogenous CRKL was immunoprecipitated from lysates of cells transfected with the indicated constructs and was immunoblotted with anti-phosphotyrosine. * indicates an HTM tag, which reduces RIN-ABD migration in comparison to panels (B) and (C). Relative CRKL phosphorylation levels are indicated at the bottom of each lane. Immunoblots confirming expression and normalized CRKL levels are shown below.

(E) Endogenous CRKL was immunoprecipitated from forebrain extracts of wild-type (wt) and *Rin1*$^{-/-}$ mice and analyzed by anti-phosphotyrosine immunoblot. Below is a CRKL immunoblot for normalization.

(F) Analysis of ABL1 and ABL2 in wild-type and *Rin1*$^{-/-}$ brain extracts (several background bands are seen with this ABL1 antibody). Expression levels were normalized with a β-tubulin probe.

dence that RIN1 alters the catalytic properties of ABL2, at least in part, independently of this phosphoactivation.

Because all of the elements required for ABL binding reside in the ABD region of RIN1 (residues 1–295), we tested whether this domain alone could stimulate tyrosine kinase activity. Indeed, we detected strong stimulation of ABL2-mediated cellular phosphotyrosine by the ABD fragment of RIN1 (Figure 4B). However, RIN1-ABD^{TM}, which cannot bind to BCR-ABL1 [32] or ABL2 (this work), did not stimulate tyrosine kinase activity (Figure 4B). To confirm that immunoblot signals were due to ABL tyrosine kinase activity, the experiment was repeated with cells treated with STI571. Most of the antiphosphotyrosine signal was eliminated (Figure 4C), although, under these conditions, some phosphorylated RIN1-ABD was still detectable. Taken together, these results indicated that RIN1 promotes activation of ABL2 through a direct interaction.

We next examined the phosphorylation of CRKL, an ABL kinase substrate. Consistent with phosphotyrosine analyses of total cells, we observed a marked increase in tyrosine phosphorylation of endogenous CRKL when ABL2 was expressed with RIN1 or with RIN1-ABD (Figure 4D). An increase in tyrosine-phosphorylated CRKL was also noted in the sample that received only RIN1-ABD (compare lanes 1 and 6), reflecting stimulation of endogenous ABL proteins.

To determine whether RIN1 is a physiological regulator of CRKL phosphorylation, we analyzed mouse forebrain tissue, where RIN1 is expressed at highest levels [33]. We detected a band corresponding to tyrosine-phosphorylated CRKL, the intensity of which was markedly reduced in a *Rin1*$^{-/-}$ tissue sample (Figure 4E). The observed change was not due to reduced ABL1 or ABL2 expression (Figure 4F). These results further support a role for RIN1 in promoting the ABL kinase-mediated phosphorylation of CRKL.

Direct Activation of ABL Tyrosine Kinase Activity

We next performed in vitro kinase assays with a consensus ABL1/ABL2-substrate peptide [37] and ABL2 protein immunoprecipitated from cell lysates. Kinase activity was detected in material from ABL2 and ABL2^{Y439F} cell lysates, but ABL2^{K319R} yielded only background activity (Figure 5A). Coexpression of RIN1-